IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benvenisty	Atty Docket:	BENVENISTY5
Serial No.:	09/995,452	Art Unit:	1632
Date Filed:	November 27, 2001	Examiner:	Ton, Thaian N.
Invention:	Transfection of Human Embryonic Stem cells		

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CERTIFICATE OF MAILING

I hereby certify that this document, along with any other papers referred to as being attached or enclosed, is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on xxxxxx.

Roger L. Browdy

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF BENVENISTY, M.D., PH.D. IN SUPPORT OF APPLICANT'S RESPONSE OF JANUARY 5, 2006 [37 C.F.R. § 1.132]

Dear Sir:

In support of the Response and Request for Continued Examination filed on January 5, 2006 in reply to the Office Action of July 5, 2005 for the above-referenced matter, I hereby declare as follows:

1. My name is Nissim Benvenisty, M.D., Ph.D. I am a Professor at The Hebrew University in Jerusalem, Israel, in the Department of Genetics. I was formerly the Vice Chair of the Institute of Life Sciences at The Hebrew University, and have been a visiting Professor in the Department of Genetics at Harvard University in Boston, MA, among other positions. I have been awarded numerous prizes and fellowships, including the Teva Prize for excellent research in stem cells in 2003, the Herbert Cohn Chair in Cancer Research during 1999, the Hestrin Prize in Molecular Biology

in 1997, a Howard Hughes Postdoctoral Fellowship from 1991-1993, and a Fulbright Postdoctoral Fellowship from 1990-1991. I have published extensively in the field of stem cell research, and other areas, with over 65 publications, to date. I am also an inventor or co-inventor of a substantial number of patents involving (among other things) human embryonic stem cell research, and I am a co-inventor of the invention claimed in the current application. My further credentials are set forth in my Curriculum Vitae, which is attached hereto as Exhibit A.

2. I have read the action of July 5, 2005. This declaration is provided to clarify the record concerning the generation of a substantially pure stably transfected population of pluripotent human embryonic stem cells, wherein said cells are modified to contain a gene expression altering sequence of DNA.

3. The Examiner contends that "Smith teach that their method provides for isolation and/or enriching and/or selectively propagating animal stem cells. Thus, these selection or enrichments steps, as taught by Smith clearly result in what the skilled artisan would consider a substantially pure population of cells." I must disagree with the Examiner's understanding of the state of the art. Since human embryonic stem cells are considerably different from mouse embryonic stem cells, the methodology of transfection in the murine cells is distinct from that in the human cells. The basis for this difference relies on two main issues as will be detailed below:

   a. Electroporation as performed in mouse ES cells is inefficient in human ES cells and alternative methodology had to be established in order to arrive at the substantially pure population of stably transfected cells.

   b. Since human ES cells, unlike the mouse cells, do not grow well in dilution, it would have been impossible to arrive at stably transfected cells using an inefficient transfection method (such as electroporation).

4. Primarily, had the Examiner's assumption been accurate, there would have been no difficulty for other researchers in the field to have succeeded in generating a substantially pure stably transfected population of pluripotent human embryonic stem cells. However, this was clearly not the case, as I will try to convey in my arguments below.

5. There are (at least) two hindering technical issues that should be considered when referring to the generation of this stably transfected population of pluripotent human embryonic stem cells, in view of what was known in the field of stem cell research, at the time of the invention. Firstly, that the transfection protocols that worked so successfully for the murine system, were not applicable to the human system. And secondly, that when human stem cells are too diluted in culture, they tend to die. I shall discuss these two issues in more detail below.

6. We have gone in length in our correspondence with the Examiner to explain the particular methodology developed in my laboratory, which enabled us to achieve the results presented in the present application, i.e., the successful transfection of hES cells with exogenous DNA, using a chemical reagent, particularly a cationic non-lipid polymer transfection reagent.

7. Nonetheless, with respect to the specific clonal population of stably transfected cells that we generated through this technology, the Examiner contends that there is no true inventive step in obtaining this population. Particularly, the Examiner supports the idea that based on the knowledge available at the time, one could have still obtained such clonal population using electroporation of hES cells, since all one needs to arrive at this result is one viable clone, and thus the fact that electroporation results in a much lower yield of transfected cells, should not interfere with the final result. In theory, the Examiner's conclusion might be correct. In practice, however, at

the laboratory bench, this is not the reality that I or my colleagues in the field encountered. Thus, I will try to express the fact that obtaining said population of stably transfected hES cells, described in the present application, was not at all trivial.

8. There is a concept of "dilution" when trying to maintain human embryonic stem clel lines. Human embryonic stem cell lines must be cultured on a culture medium with murine feeder cells. However, there is a feedback mechanism between the human stem cells and the feeder cells, amongst the human stem cells themselves, such that if the human stem cells are too diluted on the culture plate, they do not survive. I would like to refer to an article by Amit *et al.* (Amit, M. *et al.* "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture" – *Developmental Biology*; vol. 227, p.271-278, 2000; Exhibit B), where the authors describe as follows:

> To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96-well plate containing mouse embryonic fibroblast feeders with medium containing 20% serum replacer and 4 ng/ml bFGF. (page 272, top of the 2nd column)

Under these very diluted conditions, (1 cell/well), the cloning efficiency was extremely poor. And even using a serum replacer, in the presence or absence of bFGF, did not result in an efficiency above 1% (see Table 1, page 273). The authors further discuss this issue on page 277:

> Because current culture conditions are suboptimal, a significant percentage of the ES cells die at each passage, even when they are passaged in clumps.

Thus, it was a known fact in the field that individually passaged cells are even more vulnerable, and thus these are less likely to survive the cloning process. From Amit et al., it can be learned that using very diluted conditions of 1 cell/well, for example, results in extremely low (less than 1%) survival.

9. In order to reach stable transfection, which requires prolonged cloning periods, less "diluted" conditions are needed as only these will enable satisfactory cloning efficiency. In other words, in order to reach stable transfection, it is necessary, first of all, to obtain a good transfection yield, so that there is a sufficient number of cells to initiate the cloning process with. If the transfection yield is too small, then once you put the transfected cells onto culture plates with neomycin in order to kill off the non-stably transfected cells, one would be left with an extremely small number of colonies, which would very likely die because of their dilution, and the lack of cell-cell interactions necessary for their survival. Thus, the establishment of an efficient transfection protocol was essential to achieve a sufficient yield of stably transfected cells, which could then sufficiently populate the culture plate and be selected with neomycin, while avoiding the dilution problem.

10. In this regard I would like to refer to the article by Zwaka and Thomson (Zwaka, T.P. and Thomson, J.A. "Homologous recombination in human embryonic stem cells" – *Nature Biotechnology*; vol. 21, p.319-321, 2003; Exhibit C), first cited in "Declaration 1" as Exhibit C, filed on October 25, 2004.

11. Zwaka and Thomson describe homologous recombination in human embryonic stem cells, following their transfection, and state the following:

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10^{-5}, mouse ES cell electroporation procedures yield substantially lower rates.

The electroporation transfection rate in that article was reported as being only about $\sim 10^{-7}$ when mouse electroporation protocols were adapted and applied for transfection of hES cells (see Zwaka, p. 319, col. 2, first para).

12. As I said above, one would not start cloning with very low cell numbers, because one would not expect this small number of cells to produce a substantially pure, stably transfected cell line.

13. In conclusion, for the generation of our substantially pure stably transfected population of pluripotent human embryonic stem cells my laboratory overcame two major technological obstacles, transfecting human ES cells and isolating a clonal population, which should be taken into consideration when evaluating the uniqueness of said cell population.

14. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Benvenisty, M.D., Ph.D.

15. Dated: April 2nd, 2006

Exhibits:

A. *Curriculum Vitae* of Nissim Benvenisty, M.D., Ph.D.

B. Amit, M. *et al.* "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture" – *Developmental Biology*; vol. 227, p.271-278, 2000.

C. Zwaka, T.P. and Thomson, J.A. "Homologous recombination in human embryonic stem cells" – *Nature Biotechnology*; vol. 21, p.319-321, 2003.

NISSIM BENVENISTY

CURRICULUM VITAE

Date and Place of Birth : 9.10.1958; Israel.

Marital Status: Married + 3.

Education :

1983	M.D.	Faculty of Medicine, Hebrew University.
1986	Ph.D.	Department of Developmental Biochemistry, Hadassah Medical School, Hebrew University.

Employment and Related Training :

2002-	Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
2002-2003	Head of Biology Teaching and Vice Chair, Institute of Life Sciences The Hebrew University, Jerusalem, Israel
1999-2000	Visiting Professor, Department of Genetics, Harvard University, Boston, USA
1998-2002	Associate Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1993-1998	Senior Lecturer, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1990-1993	Research Fellow, Department of Genetics, Harvard Medical School, Boston, USA. Under supervision of Professor Philip Leder.
1986-1990	Israeli Army Medical Service.
1983-1986	Graduate Student, Department of Developmental Biochemistry, The Hebrew University, Jerusalem, Israel.
1985	Research Associate, Case Western Reserve University, Cleveland, USA.

EXHIBIT A

1983-1984	Internship, Hadassah Hospital, Jerusalem, Israel.
1982-1985	Teaching biochemistry and molecular biology to medical students at The Hebrew University.

Awards and Fellowships :

1981	Awarded the Faculty Prize.
1982	Awarded a Fellowship at the Mount Sinai Hospital, New York - Program for outstanding students.
1982-1985	Foulkes Foundation Fellowship.
1985	Best Teacher Award for teaching biochemistry and molecular biology.
1988	Awarded the Senta Foulkes Prize (London).
1990-1991	Awarded the Weizmann Postdoctoral Fellowship.
1990-1991	Awarded the Fulbright Postdoctoral Fellowship.
1991-1993	Awarded the Howard Hughes Postdoctoral Fellowship.
1993-1996	Awarded the Alon Fellowship.
1994	The Joseph H. and Belle R. Braun Senior Lectureship in Life Sciences.
1995-1998	Awarded Best Teacher in Genetics.
1995	Awarded the Hebrew University Prize for Young Scientist.
1996	Awarded the Naftali Prize.
1997	Awarded the Hestrin Prize in Biochemistry and Molecular Biology.
1998	Awarded the Rom Prize in Genetics
1999	The Herbert Cohn Chair in Cancer Research
1999-2000	Awarded the Yamagiwa-Yoshida Memorial International Cancer Study Fellowship.
2003	Awarded the Teva Prize for excellent research in stem cells

List of publications

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Developmental Biology 227, 271–278 (2000)
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Clonally Derived Human Embryonic Stem Cell Lines Maintain Pluripotency and Proliferative Potential for Prolonged Periods of Culture

Michal Amit,* Melissa K. Carpenter,† Margaret S. Inokuma,† Choy-Pik Chiu,† Charles P. Harris,‡ Michelle A. Waknitz,§ Joseph Itskovitz-Eldor,* and James A. Thomson§,¶

*Department of Obstetrics and Gynecology, The Rambam Medical Center, and Faculty of Medicine, Technion, Haifa 31096, Israel; †Geron Corporation, 230 Constitution Drive, Menlo Park, California 94025; ‡Texas Children's Hospital, Baylor College of Medicine, Houston, Texas 77030; and §The Wisconsin Regional Primate Research Center and ¶Department of Anatomy, School of Medicine, University of Wisconsin, Madision, Wisconsin 53715

Embryonic stem (ES) cell lines derived from human blastocysts have the developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. Here we describe the clonal derivation of two human ES cell lines, H9.1 and H9.2. At the time of the clonal derivation of the H9.1 and H9.2 ES cell lines, the parental ES cell line, H9, had already been continuously cultured for 6 months. After an additional 8 months of culture, H9.1 and H9.2 ES cell lines continued to: (1) actively proliferate, (2) express high levels of telomerase, and (3) retain normal karyotypes. Telomere lengths, while somewhat variable, were maintained between 8 and 12 kb in high-passage H9.1 and H9.2 cells. High-passage H9.1 and H9.2 cells both formed teratomas in SCID-beige mice that included differentiated derivatives of all three embryonic germ layers. These results demonstrate the pluripotency of single human ES cells, the maintenance of pluripotency during an extended period of culture, and the long-term self-renewing properties of cultured human ES cells. The remarkable developmental potential, proliferative capacity, and karyotypic stability of human ES cells distinguish them from adult cells.

Key Words: human embryonic stem cells; basic fibroblast growth factor; cloning; telomeres.

INTRODUCTION

Human pluripotent cell lines have been derived from preimplantation embryos (embryonic stem cell lines, ES cells; Reubinoff *et al.*, 2000; Thomson *et al.*, 1998) and from fetal germ cells (embryonic germ cell lines, EG cells; Shamblott *et al.*, 1998) that for prolonged periods of culture maintain a stable developmental potential to form advanced derivatives of all three embryonic germ layers. Human ES cell lines have widespread implications for human developmental biology, drug discovery, drug testing, and transplantation medicine. For example, current knowledge of the postimplantation human embryo is largely based on a limited number of static histological sections, and because of ethical considerations, the underlying mechanisms that control the developmental decisions of the early human embryo remain essentially unexplored. Although the mouse is the mainstay of experimental mammalian developmental biology, there are significant differences between early mouse and human development. These differences are especially prominent in the extraembryonic membranes, in the placenta, and in the arrangement of the germ layers at the time of gastrulation. The yolk sac, for example, is a robust, well-vascularized extraembryonic tissue that is important throughout mouse gestation, but in the human embryo, the yolk sac is essentially a vestigial structure during later gestation (Kaufman, 1992; O'Rahilly and Muller, 1987). Human ES cells should provide important new insights into the differentiation and function of tissues that differ significantly between mice and humans.

In addition to advancing basic developmental biology, human ES cells should have practical, applied uses. The differentiated derivatives of human ES cells could be used for: (1) identification of gene targets for new drugs, (2)

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EXHIBIT B

testing the toxicity or teratogenicity of new compounds, and (3) transplantation to replace cell populations destroyed by disease. Potential conditions that might be treated by the transplantation of ES cell-derived cells include Parkinson's disease, cardiac infarcts, juvenile-onset diabetes mellitus, and leukemia (Gearhart, 1998; Rossant and Nagy, 1999). However, the scientific and therapeutic potential of human ES cells critically depends on their long-term proliferative capacity, their developmental potential after prolonged culture, and their karyotypic stability. The originally described human ES and EG cell lines were not clonally derived from single cells and thus pluripotency could be demonstrated only for a population of cells (Reubinoff *et al.*, 2000; Shamblott *et al.*, 1998; Thomson *et al.*, 1998). Therefore, the formal possibility existed that within the population of homogeneous-appearing cells there were actually multiple precursor or stem cells committed to different lineages and that no single cell was capable of forming derivatives of all three embryonic germ layers. The human ES cells we previously derived were isolated and propagated as small clumps because nonhuman primate ES cell colonies dissociated to single cells plate at a very low efficiency (Thomson *et al.*, 1995; Thomson and Marshall, 1998).

Here we describe conditions for clonally deriving human ES cell lines. Two clonally derived human ES cell lines proliferated for a period of at least 8 months after clonal derivation (population doubling (PD) 286 from initial derivation of the parental cell line) and maintained the ability to differentiate to advanced derivatives of all three embryonic germ layers. Both clonal human ES cell lines expressed high levels of telomerase and maintained terminal restriction fragment (TRF) lengths between 8 and 12 kb. In contrast, normal human somatic cells gradually lose telomeric DNA and senesce after 50–80 PD when TRF lengths are about 5–7 kb. Thus, these results clearly demonstrate the pluripotency of single human ES cells and demonstrate the remarkable proliferative capacity of these cells.

MATERIALS AND METHODS

The derivation, routine culture, and characterization of the human ES cell line H9 were previously described (Thomson *et al.*, 1998). Human ES cells were plated on irradiated (35 gray γ irradiation) mouse embryonic fibroblasts. Culture medium for the present work consisted of 80% KnockOut Dulbecco's modified Eagle's medium, an optimized medium for mouse ES cells (Gibco BRL, Rockville, MD), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids stock (Gibco BRL), supplemented with either 20% fetal bovine serum (HyClone, Logan, UT) or 20% KnockOut SR, a serum replacer optimized for mouse ES cells (Gibco BRL). The components of KnockOut SR have been published elsewhere (Price *et al.*, 1998). In initial cloning experiments, medium was supplemented with either serum or serum replacer and was used either with or without human recombinant basic fibroblast growth factor (bFGF; 4 ng/ml). For prolonged culture, the serum-free medium required supplementation with bFGF.

To determine cloning efficiency, H9 cells were dissociated to single cells for 7 min with 0.05% trypsin/0.25% EDTA, washed by centrifugation, and plated on mitotically inactivated mouse embryonic fibroblasts (10^5 ES cells in triplicate wells of 6-well plates). To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96-well plate containing mouse embryonic fibroblast feeders with medium containing 20% serum replacer and 4 ng/ml bFGF. Clones were expanded by routine passage every 7 days with 1 mg/ml collagenase type IV (Gibco BRL).

For karyotype analysis, either standard G banding or multicolor spectral karyotyping (SKY) was performed (Schrock *et al.*, 1996). For SKY analysis, the SKY H-10 kit was used according to the manufacturer's instructions (Applied Spectral Imaging, Inc., Carlsbad, CA). Metaphase figures were dropped onto clean glass slides and treated with combinatorially labeled whole genome painting probes. After stringent washes in 50% formamide, images of metaphase spreads were captured using the Applied Spectral Imaging spectrophotometer and SKY software on a Zeiss Axioplan II microscope. Karyotypes were analyzed and arranged with combined software processing of the image and reverse DAPI banding. For each SKY sample, 5 metaphases were captured and analyzed completely, and 20 metaphases were captured for modal number determination.

Prior to measurement of telomerase activity and telomere length, ES cells expressing TRA-1-60 (a marker of undifferentiated human ES cells) were selected from cultures grown on irradiated mouse embryonic fibroblasts. Cells were dissociated using 0.2% EDTA and then incubated with a monoclonal antibody against TRA-1-60 (gift from Peter Andrews). After washing, cells were incubated with goat anti-mouse IgM-conjugated magnetic microbeads and processed through a MACS magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) (Walz *et al.*, 1995). In the samples used for these experiments, greater than 90% of the population was positive for TRA-1-60 using flow cytometric analysis. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) assay as described (Kim *et al.*, 1994; Weinrich *et al.*, 1997). TRF size was determined using Southern hybridization essentially as described (Allsopp *et al.*, 1992; Harley *et al.*, 1990; Vaziri *et al.*, 1993).

For teratoma formation, H9.1 and H9.2 cells, cultured for 6 months after cloning, were injected into the rear leg muscle of 4-week-old male SCID-beige mice (eight mice total). Cell numbers ranged from 2.5×10^6 cells to 7.5×10^6 cells per injection. Three to four months after injection the mice were sacrificed and the resulting teratomas examined histologically.

RESULTS

To demonstrate the long-term pluripotency and replicative immortality of single human ES cells, we have derived clonal human ES cell lines. These clonal lines have been maintained for over 1 year *in vitro*, and proliferation rate, karyotypes, teratoma formation, telomere length, and telomerase activity have been examined.

The cloning efficiency of human ES cells was extremely poor under previously described culture conditions that included serum. A several-fold increase in cloning efficiency of human ES cells was consistently observed when serum-free medium was used instead of serum-containing

TABLE 1
Cloning Efficiency of H9 Human ES Cells[a]

	(−) bFGF	(+) bFGF (4 ng/ml)
20% Serum	240 ± 28 (0.24)*	260 ± 12 (0.26)*
20% Serum replacer	633 ± 43 (0.63)†	826 ± 61 (0.83)‡

[a] Values are expressed as the mean numbers of colonies resulting from 10^5 individualized ES cells plated ±SE (figures in parentheses represent percentage colony cloning efficiency).

*·†·‡ Means with different superscripts differ significantly ($P < 0.05$, Tukey-Kramer HSD.

medium (Table 1). The addition of bFGF to the medium altered the morphology of human ES cells, resulting in smaller cells in tighter colonies (Fig. 1A). The long-term culture of human ES cells in the presence of serum does not require the addition of exogenous bFGF and the addition of bFGF to serum-containing medium did not significantly increase human ES cell cloning efficiency (Table 1). However, in serum-free medium, bFGF increased the initial cloning efficiency of human ES cells and bFGF was required for continued undifferentiated proliferation. In serum-free medium lacking bFGF, human ES cells became uniformly differentiated by 2 weeks after plating (Fig. 1B).

To avoid the possibility that some of the colonies that grew were not from individual cells, but were from rare, small clumps of cells remaining after dissociation, the H9 cell line was recloned by placing cells individually into wells of a 96-well plate under direct microscopic observation. Of 384 H9 cells (at PD 122) plated into 96-well plates, 2 clones were successfully expanded (H9.1 and H9.2). Both of these clones were subsequently cultured continuously in medium supplemented with serum replacer and bFGF. H9.1 and H9.2 cells maintained a normal XX karyotype even after more than 8 months of continuous culture after clonal derivation (Fig. 2).

Population doubling time was measured in the parent line and in both clonal lines and no significant differences were found between the parent line and the clonal lines. The average population doubling time for 10 separate determinations was 35.3 ± 2.0 h (mean ± standard error of the mean). Because of the considerable cell death observed in these human ES cell cultures, this population doubling time may underestimate the replication rate of the ES cells that survive. In addition, evaluation of the karyotype in the H9 parental population 6 months after derivation (PD 122) presented a normal XX karyotype by standard G-banding techniques (20 chromosomal spreads analyzed). However, 7 months after derivation, in a single karyotype preparation, 4/20 spreads demonstrated random abnormalities; one with a translocation to chromosome 13 short arm, one with an inverted chromosome 20, one with a translocation to the number 4 short arm, and one with multiple fragmentation. Subsequently, at 8, 10, and 12.75 months after derivation (PD 260), H9 cells exhibited normal karyotypes in all 20 chromosomal spreads examined. Both H9.1 and H9.2 also exhibited normal karyotypes 8 months after derivation.

Telomerase activity was high in H9, H9.1, and H9.2 cells at all time points examined (Fig. 3), ranging from about 52 to 196% of that found in H1299, a lung tumor cell line. In these experiments, the human ES cells were separated from fibroblast feeders by magnetic bead sorting to greater than 90% of the cells as determined by Tra-1-60 expression. Even though telomerase activity was also found in the mouse feeder cells (MEF, Fig. 3), the activity in MEF is 4–15% of that found in human ES cells. Therefore, it is unlikely that

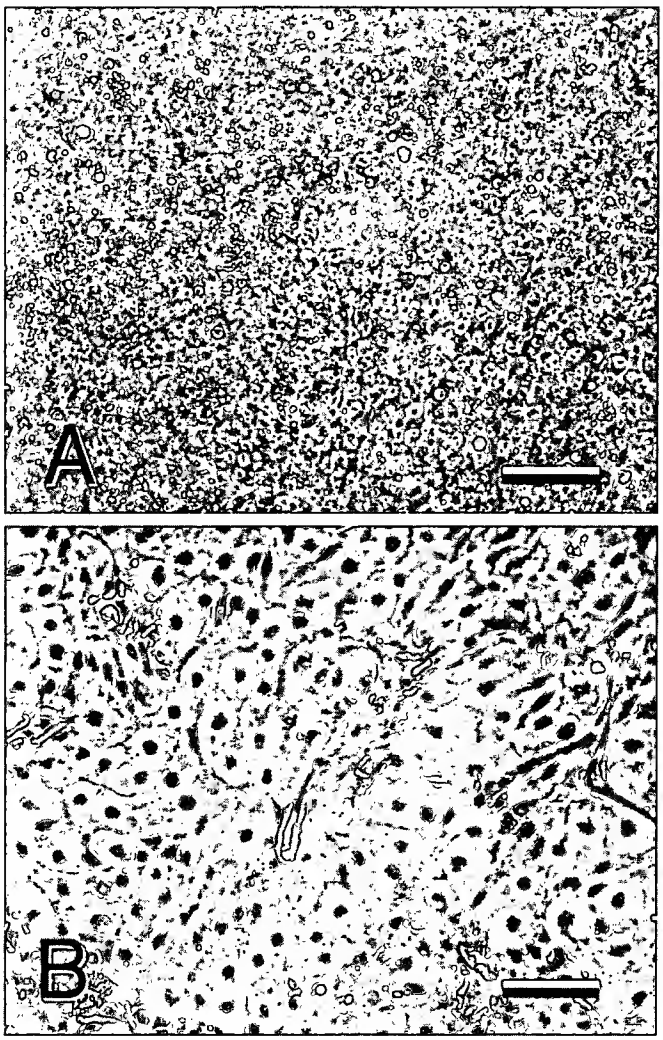

FIG. 1. H9 human ES cells cultured for 14 days in serum-free medium in the presence (4 ng/ml) (A) or absence (B) of bFGF. ES cells plated in serum-free medium in the presence of bFGF continued active, undifferentiated proliferation throughout the culture period. ES cells plated in the absence of bFGF uniformly differentiated into a flattened, epithelial morphology by the end of the 14-day culture period. Bar, 200 μm.

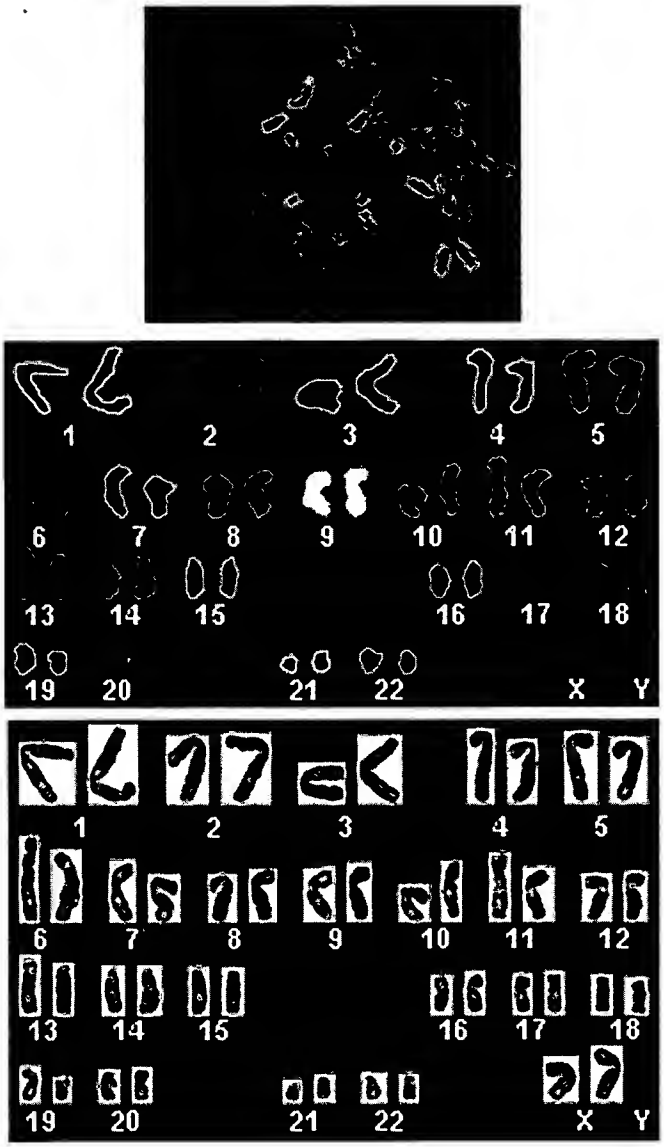

FIG. 2. Metaphase preparation from H9.2 at 8 months (PD 175) of continuous culture. Captured metaphase spectral image (top); classified processed image (middle); reverse DAPI image (bottom). Karyotype: 46, XX normal female.

the telomerase activity seen in the human ES cells is entirely due to the <10% contamination from the feeders.

In general, the presence of telomerase activity is associated with a maintenance of telomere length. H9.1 and H9.2 clones showed roughly stabilized telomere lengths, between 8 and 12 kb. As seen in other populations of telomerase-positive cells, changes in telomere length did not correlate with telomerase activity. At PD 57 after clonal derivation, H9.2 cells had a telomere length of about 8 kb which increased to 13 kb by PD 143. H9.1 cells showed an initial decrease in telomere length followed by an increase to about 11 kb at PD 123. On the other hand, the parental cell line, H9, demonstrated an initial decrease in telomere length from 14 kb at PD 71 (passage 15) to 9 kb at PD 200 (passage 42) (Fig. 3). Cells at later PD will need to be examined to determine if telomeres continue to shorten. However, to date, H9 is still proliferating well at PD 304.

The H9.1 and H9.2 clones maintained the potential to form derivatives of all three embryonic germ layers after long-term culture under serum-free conditions. After 6 months of culture (PD 122), H9.1 and H9.2 clones were confirmed to have normal karyotypes and were then injected into SCID-beige mice. Both H9.1 and H9.2 cells formed teratomas that contained derivatives of all three embryonic germ layers, including gut epithelium (endoderm); embryonic kidney, striated muscle, smooth muscle, bone, and cartilage (mesoderm); and neural tissue (ectoderm) (Fig. 4). The range of differentiation observed within the teratomas of the high-passage H9.1 and H9.2 cells was comparable to that observed in teratomas formed by low-passage parental H9 cells.

DISCUSSION

This report addresses two critical issues: the pluripotentiality and the long-term replicative capacity of clonal human ES cell lines. The previously described human ES cell lines appeared to be a morphologically homogeneous population of cells and expressed cell surface markers characteristic of clonally derived primate ES cells (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998, 1995; Thomson and Marshall, 1998). However, because these human ES cell lines were not clonally derived, pluripotency could be demonstrated only for a population of cells and not for individual ES cells. Our successful cloning of human ES cell lines demonstrates that individual cells are indeed pluripotent and that such individual pluripotent ES cells were present after at least 6 months of continuous culture of the parental H9 cell line. Furthermore, these clonally derived cell lines demonstrated a developmental potential after more than 6 months of additional culture (12 months after the derivation of the parental cell line) that was comparable to low-passage ES cells.

The telomere hypothesis suggests that critical telomere shortening signals cell senescence (Harley *et al.*, 1990; Vaziri *et al.*, 1993) and that telomerase can prevent senescence by maintenance of telomere length (Bodnar *et al.*, 1998; Jiang *et al.*, 1999). This hypothesis is supported by the presence of high telomerase activity in cells with extensive replicative capacity such as germ cells or tumor cells (Chiu and Harley, 1997). The telomerase activity seen in human ES cells, especially in the cell lines which have survived approximately 300 population doublings, i.e., four to six times the life span of normal somatic cells, suggests that these cells are immortal.

Telomere lengths in cloned human ES cells showed some variations from passage to passage, but are stabilized over-

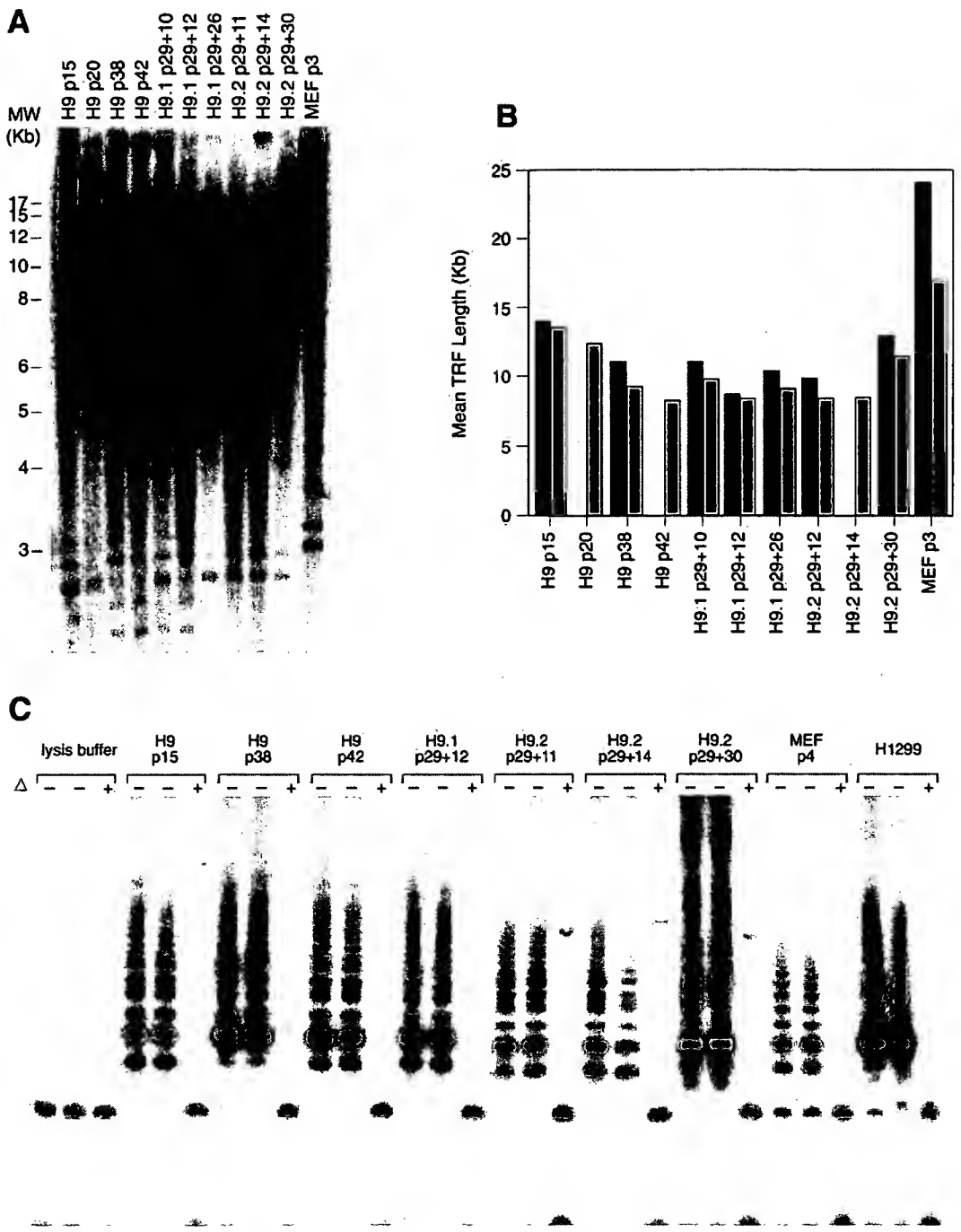

FIG. 3. Telomere length and telomerase activity in human ES cells. (A) Southern analysis of terminal restriction fragment (TRF) from the H9 parent line and two clonal lines, H9.1 and H9.2, at different times *in vitro*. Passage number for the clones is represented as number of passages following subcloning at passage 29 (p29 + *X*). Cells were passaged at 7-day intervals. MEF indicates irradiated mouse primary embryonic fibroblasts. (B) Mean TRF lengths. Telomere length was quantified using a PhosphorImager and ImageQuant software. The mean TRF length for each lane is an integral function based on the densitometric readings in reference to the standards for each gel (A). Mean TRF length was calculated from replicate analyses: light gray bars represent data for gel A, dark gray bars represent replicate analysis of selected samples from gel A. (C) TRAP analysis of telomerase activity in human ES cells. The TRAP assay is a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. The telomerase extension products serve as a template for PCR amplification. The laddering in the TRAP gel represents increasing numbers of telomeric repeats in which radionucleotides are incorporated. Samples were run in triplicate with the third sample as a negative control in which cell lysates were heat inactivated prior to the assay (indicated by the Δ). The positive control was a cell extract from the telomerase-expressing tumor line H1299 cells.

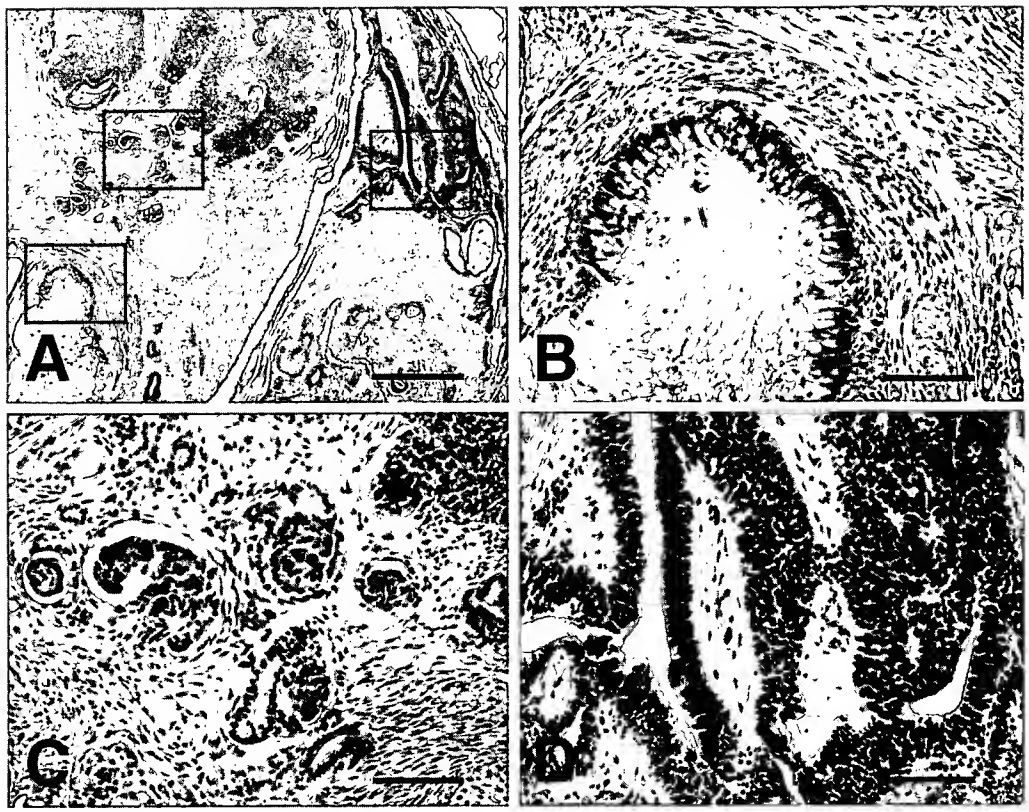

FIG. 4. Teratomas from H9.1 cells. Approximately 5×10^6 H9.1 cells that had been cultured for 6 months (PD 131) after clonal derivation were injected into the hind leg musculature of a SCID-beige mouse. The resulting tumor was harvested and examined 3 months after injection. (A) Low-power view of a field that exhibits differentiated derivatives of all three embryonic germ layers. Areas enclosed in boxes are enlarged in B, C and D (bar, 500 μm). (B) Gut epithelium with adjacent smooth muscle (bar, 100 μm). (C) Embryonic glomeruli and renal tubules (bar, 100 μm). (D) Neural epithelium (bar, 100 μm).

all. This could be due to heterogeneity in the differentiated state of the cultures or could be due to variation or drift in the abundance of factors which establish the equilibrium point for telomere length, in which gain due to telomerase activity matches loss due to incomplete replication and degradation (Chiu *et al.*, 1996; Vaziri *et al.*, 1994). The parental H9 ES cell line showed a decrease in telomere length with increasing population doublings. Despite the telomere shortening, H9 cells are still proliferating well at PD 304. Other immortalized cells also demonstrate telomere shortening in the presence of telomerase and continued proliferation. For example, endothelial cells engineered to express telomerase continue to proliferate but show decreased telomere length which eventually stabilizes (Yang *et al.*, 1999). We are continuing to monitor telomere length in H9 cells.

Serum is a complex mixture that can contain compounds both beneficial and detrimental to human ES cell culture. Different serum batches vary widely in their ability to support vigorous undifferentiated proliferation of human ES cells. Replacing serum with defined components should reduce the variability of experiments associated with serum batch variation and should allow more carefully defined differentiation studies. The consistently lower cloning efficiency in medium containing serum suggests the presence of compounds that are detrimental to stem cell survival when the cells are dispersed to single cells. Although we were able to sustain human ES cells for prolonged periods in a commercially available serum substitute, substantial improvements to the serum-free culture of human ES cells are still needed. Most important, the present culture conditions support a cloning efficiency of human ES cells (<1%) that is considerably lower than the cloning efficiency of mouse ES cells. Unless cloning efficiency is increased significantly, techniques standard to mouse ES cells, such as homologous recombination, will be very difficult to apply to human ES cells.

The fibroblast feeder layer remains the most poorly defined component of the human ES cell culture environment. Unlike undifferentiated mouse ES cells, which can be cultured using leukemia inhibitory factor (LIF) in the absence of fibroblasts (Smith *et al.*, 1988; Williams *et al.*,

1988), under current culture conditions, addition of LIF to the medium does not allow the culture of human ES cells in the absence of feeder layers (Thomson *et al.*, 1998). If LIF and bFGF are added in combination, human ES cells are still lost to differentiation in the absence of fibroblasts (not shown). To date we have also been unable to demonstrate beneficial effects of exogenously added LIF in the presence of fibroblasts (not shown). However, fibroblasts can produce LIF, and given the importance of LIF in the culture of human EG cells (Shamblott *et al.*, 1998), further examination of the role of LIF in human ES cell self-renewal is warranted. Identifying the factors that the fibroblasts produce that promote human ES cell renewal will be critical to the large-scale growth of ES cells, because the feeder layers are labor intensive to prepare and because variation between batches of fibroblasts can introduce undesirable variation and complexity to experiments. It is unknown, for example, whether the beneficial effects of bFGF on undifferentiated human ES cell growth are mediated through the fibroblasts, the ES cells, or both.

Because current culture conditions are suboptimal, a significant percentage of the ES cells die at each passage, even when they are passaged in clumps. For this reason, there is selective pressure for any rare cell population that has an enhanced survival at splitting or an increased rate of proliferation. Improved passaging and culture conditions that increase cloning efficiency should reduce this selective pressure. Given this selective pressure, the karyotypic stability of human ES cells is remarkable, but clones with abnormal karyotypes and a selective growth advantage should eventually take over a culture. Therefore, it is likely that it will be necessary to periodically subclone human ES cells to maintain a euploid population. In a single sample of H9 cells at 7 months of culture, we observed a subpopulation (4 of 20 chromosomal spreads) with abnormal karyotypes. These were the only abnormal karyotypes we observed, and curiously, subsequent karyotypes of the uncloned H9 cells at 8, 10, and 12.75 months of culture revealed 20 of 20 normal chromosomal spreads. We have yet to observe abnormal karyotypes in the H9.1 or H9.2 cells, even at 8 months after their clonal derivation. Thus, although the useful long-term expansion of human ES cells could be somewhat limited by a need to subclone periodically, our results suggest that this need will be infrequent. Ultimately, the slow accumulation of genetic mutations may place an upper limit on the useful long-term culture of human ES cells. Although these considerations may place some practical constraints on the maintenance of human ES cells, the potential uses of ES cells as biological research tools and as therapeutic agents remain unparalleled.

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Homologous recombination in human embryonic stem cells

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Homologous recombination applied to mouse embryonic stem (ES) cells has revolutionized the study of gene function in mammals[1–4]. Although most often used to generate knockout mice, homologous recombination has also been applied in mouse ES cells allowed to differentiate *in vitro*. Homologous recombination is an essential technique if human ES cells[5] are to fulfill their promise as a basic research tool. It also has important implications for ES cell–based transplantation and gene therapies. Significant differences between mouse and human ES cells have hampered the development of homologous recombination in human ES cells. High, stable transfection efficiencies in human ES cells have been difficult to achieve, and, in particular, electroporation protocols established for mouse ES cells work poorly in human ES cells[6]. Also, in contrast to their murine counterparts, human ES cells cannot be cloned efficiently from single cells, making it difficult to screen for rare recombination events[7]. Here we report an electroporation approach, based on the physical characteristics of human ES cells, that we used to successfully target *HPRT1*, the gene encoding hypoxanthine phosphoribosyltransferase-1 (HPRT1), and *POU5F1*, the gene encoding octamer-binding transcription factor 4 (Oct4; also known as POU domain, class 5, transcription factor 1 (POU5F1)).

The *HPRT1* gene is located on the X chromosome, so a single homologous recombination event leads to complete loss of function in XY cells. HPRT1-deficient cells can be selected based on their resistance to 2-amino-6-captopurine (6-TG), and thus the frequency of homologous recombination events is easy to estimate[8]. Because of these properties, HRPT1 played an important role in the initial development of homologous recombination in mouse ES cells[4,9]. We designed an *HPRT1*-targeting vector that contains a short homologous arm (1.9 kb) on the 5′ side of exon 7 and a long homologous arm (10 kb) on the 3′ side of exon 9, which deletes regions of the last three exons (Fig. 1A). A neomycin resistance (*neo*) cassette was inserted between the two homologous arms, and at the end of the 3′ homologous arm, the thymidine kinase gene (*tk*) was added to allow negative selection with gancyclovir.

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10^{-5}; mouse ES cell electroporation procedures yield substantially lower rates[6]. Given the very low transfection rates previously reported for electroporation, we first tested two chemical transfection reagents (ExGen 500 versus FuGene-6) for homologous recombination of the *HPRT1* locus in human ES cells. Although clones were obtained using both transfection reagents—for ExGen and FuGene respectively, 130 versus 261 G418-resistant clones and 35 versus 61 gancyclovir-resistant clones—none of these were resistant to both G418 and 6-TG (*HPRT1*$^-$), indicating that none were the result of homologous recombination. These results are consistent with the observation that transfection using lipid (FuGene-6; Roche, Indianapolis, IN) and cationic (ExGen 500; Fermentas, Hanover, MD) reagents results in inefficient homologous recombination in other mammalian cell types, and that physical methods of introducing DNA are, in general, more effective[10].

Our failure to achieve homologous recombination with chemical transfection reagents led us to re-evaluate electroporation procedures for human ES cells. In our hands, electroporation using a typical mouse ES cell protocol[11] (220 V, 960 μF, electroporation in PBS) yielded a stable transfection rate of ~10^{-7}. Given current culture techniques, this frequency is too low to be practical for identifying rare homologous recombination events. As human ES cells are significantly larger than mouse ES cells (~14 μm versus ~8 μm), we tried electroporation parameters described for larger cells. Also, because our current culture conditions allow only about 1% of individual human ES cells to survive and form colonies when plated at low densities, we electroporated the ES cells in clumps, not as individual cells, and plated them out at high densities. Additionally, we electroporated the cells in an isotonic, protein-rich solution (standard cell culture medium), instead of PBS, at room temperature. Using this modified protocol, we were able to obtain stable, G418-resistant clones at transfection rates that were 100-fold (or more) higher than those attained with standard mouse ES cell electroporation procedures. After transfection of 1.5×10^7 cells with the linearized *HPRT1*-targeting vector, we obtained 350 G418-resistant clones. Of these, 50 were resistant to gancyclovir, and of these, 7 were also resistant to 6-TG, suggesting successful homologous recombination. Polymerase chain reaction (PCR) and Southern blotting (Fig. 1B) confirmed that homologous recombination had occurred in all of these 6-TG-resistant clones.

One of the uses of homologous recombination in human ES cells will be to generate 'knock-in' cell lines with a selectable marker introduced into a locus with a tissue-specific expression pattern. Such knock-ins will be useful, for example, to purify a specific ES cell–derived cell type from a mixed population[12,13]. To test this approach, we introduced two reporter genes into the Oct4-encoding gene *POU5F1* by homologous recombination. Oct4, which belongs to the POU (Pit, Oct, Unc) family of transcription factors[14], is expressed exclusively in the pluripotent cells of the embryo and is a central regulator of pluripotency[14,15]. We introduced two promoterless reporter-selection cassettes into the 3′ untranslated region (UTR) of *POU5F1*. The first cassette contained an internal ribosomal entry site (IRES) sequence of the encephalomyocarditis virus and the gene *EGFP*, encoding the enhanced green fluorescence protein (EGFP). The second cassette included the same IRES sequence and the gene *neo*, encoding neomycin resistance. The cassettes were flanked by two homologous arms (Fig. 2A). After electroporation of 1.5×10^7 human ES cells with the linearized targeting vector (Fig. 2A), we obtained 103 G418-resistant clones. PCR (Fig. 2B, left) and DNA Southern blotting (Fig. 2B, right) demonstrated that 28 of these clones (27%) were positive for homologous recombination. Using a second targeting vector with a longer 3′ homologous arm, we obtained a higher rate of homologous recombination, almost 40% (22 homologous clones out of 56 G418-resistant clones). Similar transfection experiments using FuGene-6 with the same *POU5F1*-targeting vector resulted in 11 G418-resistant clones, none of which resulted from homologous recombination.

Human ES cells with the *POU5F1* knock-in expressed EGFP (Fig. 2C), which turned off during differentiation. Both drug selection and flow cytometry to detect EGFP expression (Fig. 2D) allowed purification of undifferentiated ES cells from a mixed, partially differentiated cell population. These properties will make the knock-in cell lines useful for studying *POU5F1* gene expression during differentiation *in vitro* and for optimizing culture conditions for human ES cells.

Electroporation of human ES cells with a DNA construct containing a *neo* cassette under the control of the *tk* promoter yielded a stable

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EXHIBIT C

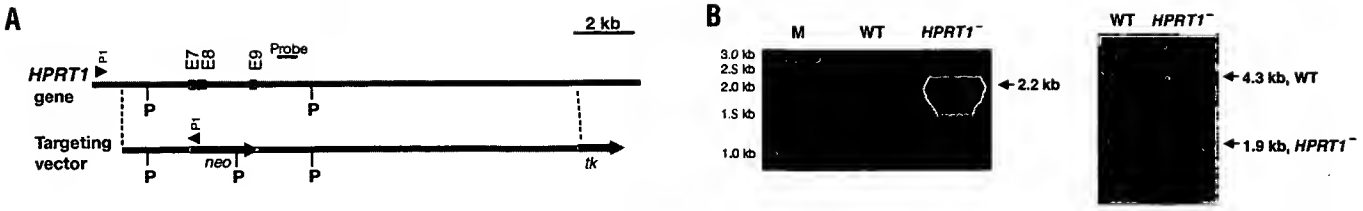

Figure 1. Targeted deletion of the last three exons of the *HPRT1* gene. (A) Partial structure of human *HPRT1* and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; P, *Pst*I; P1, primer pair; *tk*, thymidine kinase gene. (B) PCR analysis of ES cell lines with P1 (left) and Southern blot analysis with dedicated probe and *Pst*I digest (right). *HPRT1*⁻, knockout; WT, wild-type cells; M, marker.

transfection rate of 5.6×10^{-5}, giving an estimated 26:1 ratio of stable transfected clones to homologous recombination events for the first *POU5F1* construct. Similarly, for transfection of the *HPRT1* vector, the ratio of G418-resistant clones to HPRT1⁻ clones was 50:1. These targeting ratios for both HPRT1 and Oct4 are comparable to those observed for mouse ES cells[16], and suggest that although successful transfection strategies differ between human and mouse ES cells, the frequency of homologous recombination itself may be similar. However, it will be important to determine whether this similarity of rates between human and mouse ES cells holds true for genes not expressed in ES cells.

Homologous recombination in human ES cells will be important both for elucidating gene function *in vitro* and for modifying specific ES cell–derived tissues for therapeutic applications in transplantation medicine. For therapeutic applications, controlled modification of specific genes should be useful for purifying specific ES cell–derived differentiated cell types from a mixed population, for altering the antigenicity of cells, and for giving cells new properties (such as viral resistance) to combat specific diseases. Homologous recombination in human ES cells might also be used for recently described approaches combining therapeutic cloning with gene therapy[17]. Modifying specific genes for *in vitro* studies will be important for learning more about the pathogenesis of diseases for which mouse models have proven inadequate. For example, Hprt1-deficient mice do not show a phenotype similar to Lesch-Nyhan syndrome, the condition that results from HPRT1 deficiency in humans[18]. *In vitro* neural differentiation of HPRT1⁻ human ES cells or transplantation of ES cell–derived neural tissue to an animal model[19] could help clarify the pathogenesis of Lesch-Nyhan syndrome. Indeed, homologous recombination and human ES cells offer a promising approach for understanding the function of any human gene, and this approach will be particularly important for human genes that differ in clinically significant ways from the corresponding mouse genes.

Experimental protocol

***HPRT1* knockout.** The gene-targeting vector was constructed by replacement of the last three exons (exon 7, 8, and 9) of the *HPRT1* gene with a *neo* cassette under the control of the *tk* promoter. This cassette is flanked in the 5′ direction by a 10 kb homologous arm and in the 3′ direction by a 1.9 kb homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. One week before electroporation, cells were plated onto Matrigel (Becton Dickinson, San Jose, CA) and cultured with fibroblast-conditioned medium[20]. To remove colonies as intact clumps, human ES cell cultures were treated with collagenase

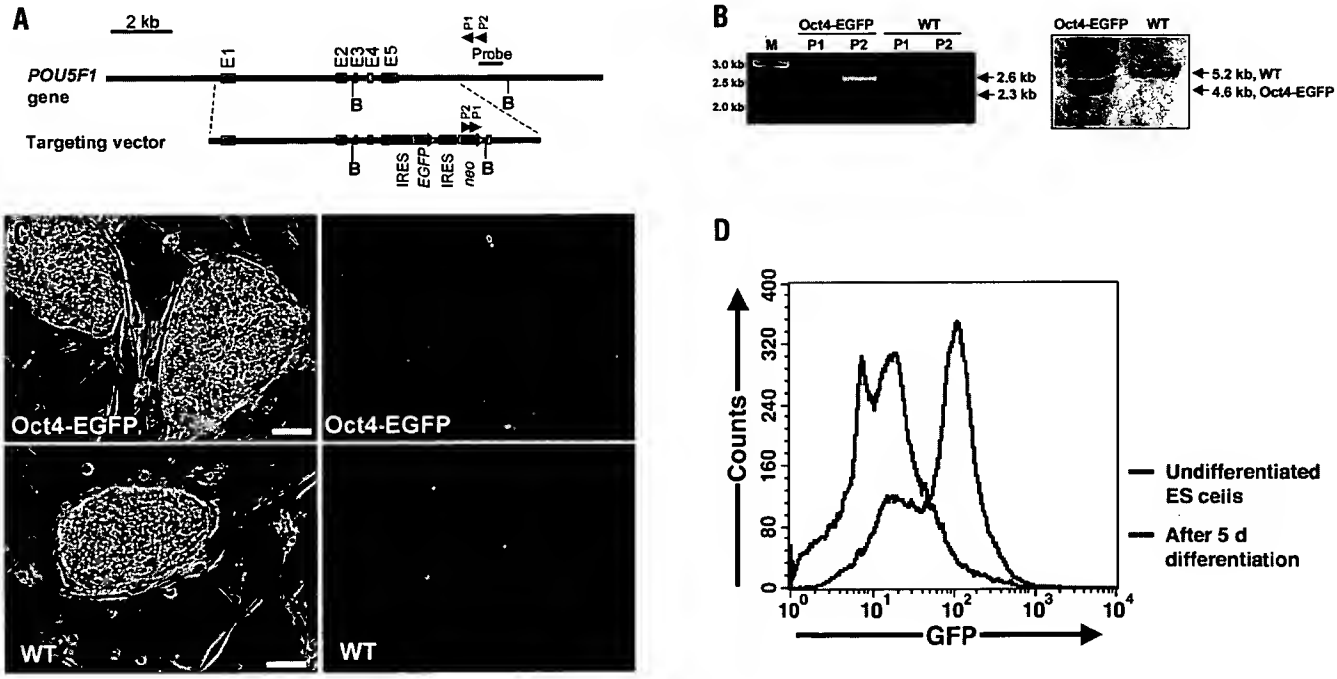

Figure 2. Targeting of an IRES-*EGFP*-IRES-*neo* cassette into the 3′ UTR of the gene *POU5F1*, which encodes Oct4. (A) Partial structure of the human *POU5F1* gene and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; B, *Bam*HI; P1 and P2, primer pairs 1 and 2. (B) PCR analysis of ES cell lines with P1 and P2 (left) and Southern blot analysis with dedicated probe and *Bam*HI digest (right). *POU5F1-EGFP*, heterozygous knock-in; WT, wild-type cells; M, marker. (C) Fluorescence microscopy (right) and phase-contrast microscopy (left) of *POU5F1* knock-in and wild-type colonies. Bar, 25 μm. (D) Flow cytometry of *POU5F1* knock-in undifferentiated (EGFP-positive) ES cells (blue) and their differentiated derivates after 5 d of differentiation (red).

IV (1 mg/ml; Invitrogen, Carlsbad, CA) for 7 min, washed with medium, and resuspended in 0.5 ml culture medium ($1.5–3.0 \times 10^7$ cells). Just before electroporation, 0.3 ml PBS (Invitrogen) containing 40 μg linearized targeting vector DNA was added. Cells were then exposed to a single 320 V, 200 μF pulse at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad, Hercules, CA). Cells were incubated for 10 min at room temperature and were plated at high density on one 10 cm culture dish coated with Matrigel. G418 selection (50 μg/ml, Invitrogen) was started 48 h after electroporation. After one week, G418 concentration was doubled and 6-TG selection (1 mM; Sigma, St. Louis, MO) was started. After three weeks, surviving colonies were analyzed individually by PCR using primers specific for the *neo* cassette and for the *HRPT1* gene just upstream of the 5′ homologous region, respectively. PCR-positive clones were rescreened by Southern blot analysis using *Pst*I-digested DNA and a probe on the 3′ side of the *neo* cassette.

POU5F1 knock-in. The gene-targeting vector was constructed by insertion of an IRES-*EGFP*, an IRES-*neo*, and an SV40 polyadenylation sequence (approximately 3.2 kb) into the 3′ untranslated region of the fifth exon of the human *POU5F1* gene. This cassette is flanked in the 5′ direction by a 6.3 kb homologous arm and in the 3′ direction by a 1.6 kb (6.5 kb in an alternative targeting vector) homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. When an alternative targeting vector with a longer (6.5 kb) 3′ homologous arm was used, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 stable clones).

Flow cytometry. Before flow cytometry, ES cell differentiation was induced by incubating the cells for 5 d in unconditioned medium on Matrigel. ES cells were treated with trypsin-EDTA and washed with PBS (both from Invitrogen). Dead cells were excluded from analysis by forward- and side-scatter gating. Samples were analyzed using a FACScan (Becton Dickinson) flow cytometer and Cellquest software (Becton Dickinson). A minimum of 50,000 events was acquired for each sample.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Site-specific cassette exchange and germline transmission with mouse ES cells expressing ϕC31 integrase

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Currently two site-specific recombinases are available for engineering the mouse genome: Cre from P1 phage[1,2] and Flp from yeast[3,4]. Both enzymes catalyze recombination between two 34–base pair recognition sites, *lox* and *FRT*, respectively, resulting in excision, inversion, or translocation of DNA sequences depending upon the location and the orientation of the recognition sites[5,6]. Furthermore, strategies have been designed to achieve site-specific insertion or cassette exchange[7–10]. The problem with both recombinase systems is that when they insert a circular DNA into the genome (*trans* event), two *cis*-positioned recognition sites are created, which are immediate substrates for excision. To stabilize the *trans* event, functional mutant recognition sites had to be identified[8–12]. None of the systems, however, allowed efficient selection-free identification of insertion or cassette exchange. Recently, an integrase from *Streptomyces* phage ϕC31 has been shown to function in *Schizosaccharomyces pombe*[13] and mammalian[14,15] cells. This enzyme recombines between two heterotypic sites: *attB* and *attP*. The product sites of the recombination event (*attL* and *attR*) are not substrates for the integrase[16]. Therefore, the ϕC31 integrase is ideal to facilitate site-specific insertions into the mammalian genome.

Here we demonstrate that the ϕC31 integrase system is compatible with embryonic stem (ES) cell–mediated genomic alterations in the mouse and is particularly useful to achieve site-specific transgene insertions or

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